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Screening And Detection Of Antiviral Activity In *Alchornea Cordifolia* Against Herpes Simplex

Virus Type 2 In Vitro

A Thesis

Presented to the Faculty of the

Department of Biology

West Chester University

West Chester, Pennsylvania

In Partial Fulfillment of the Requirements for

the Degree of

Master of Science in Biology

By

Abigail A. Yenser

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Abstract

Alchornea cordifolia, known as Pennah, has been used extensively as a medicinal plant in Africa for treating a variety of ailments. Crude water (H₂O) and methanol (MeOH) extracts prepared from dried leaves of Pennah were tested for antiviral activity against Herpes simplex virus type 2 (HSV2) by plaque reduction assays, showing crude MeOH more effective as an antiviral against HSV2. Assays with fractionated MeOH extract resulted in highest plaque reduction in the 30% MeOH fraction (30% Pennah MeOH) at a significant 98% reduction at 250 µg/mL. Crude and 30% Pennah MeOH were found to contain alkaloids, phenols, and terpenoids.

Immunofluorescence technique was utilized to visualize the extent viral products were synthesized in infected Vero cells treated with 30% Pennah MeOH extract according to different times of infection. Indirect immunofluorescence assays used to observe the course of HSV2 infection in the Vero cells in conjunction with time-sensitive and neutralization assays uncovered the extract did not prevent total viral attachment, but showed inhibition of viral replication. The specific step after viral attachment and penetration inhibited is still unknown, requiring further investigation. Time sensitive assays suggest extract must be in the presence of unattached viral particle to inhibit the viral replication due to decreased plaque reduction when extract is removed in comparison to applied to the cells at 2 and 8 hours post attachment.

Overall, *Alchornea cordifolia* exhibited antiviral activity against HSV2, having the highest potential for plaque reduction in the 30% Methanol fraction, yet the inhibited events following attachment are still undefined.

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Chapter 1

Introduction

According to the World Health Organization (47) about 13% of people aged 15-49 worldwide are infected with HSV2 and it cannot be cured, only treated with antiviral drugs. The WHO goes on to explain HSV2 is primarily sexually transmitted and can be asymptomatic or cause painful blister/ulcers at the infection site. The shedding of HSV2 is episodic and varies by individual, and sometimes infection can go unrecognized (38). This can result in spreading of the virus unknowingly by asymptomatic carriers, or individuals who present the infection with mild to no symptoms. The National Center for Health Statistics reports that more women are suffering from this infection than men, which is cause for concern when considering the possibility of spread during birth resulting in neonatal herpes (25). Another potential complication of HSV2 infection is the increased risk of acquiring HIV-1 when coupled with the viral shedding of HSV2 (47). It is found that HSV2 infection directly increases the susceptibility of an individual to HIV-1 by affecting the function of Langerhan cells even when an individual is not exhibiting symptoms (24). HSV2 remains latent and has no cure. It is a lifelong infection that individuals have to deal with and address for the rest of their lives. This leads to another aspect of HSV2 infection, which is psychosocial impact. The stigma surrounding HSV2 can result in many complications outside of the symptoms presented due to infection such as painful blisters and ulcers (47). The recurrent nature of the virus lends to the increased stigma placed on those who are infected having the possibility of causing distress, lower self-esteem, and depression (28). It is also noted many individuals feel anxiety surrounding disclosure to future partners which aids to the continued spread of the virus due to failure to disclose the risk of infection to individuals

not yet exposed (6). Due to the widespread nature of HSV2, the lifelong infection resulting from latency and flares, the stigma attached and resulting behavior, and the complications that can arise, HSV2 infection is cause for notable concern and necessity of treatment options as well as possible preventatives.

Plants have been used to treat various diseases and ailments since civilization arose, and continues to be utilized (17). *Alchornea cordifolia*, commonly known as Pennah, is a plant widely found in Africa and used in traditional medicine (8). Some common uses in medicine include antibacterial, antidiarrheal, anti-inflammatory, and pain management, as well as against malaria and fevers (8). Common ailments treated are venereal diseases such as gonorrhea, urinary tract infections, cough, sore throat, ulcers and inflammation (8). Though research has been done into antibacterial, antiplasmodial, and antifungal uses for this plant, antiviral research has yet to be accomplished. The very little work completed in antiviral studies with Pennah was with HIV-1 and HIV-2 infections and showed that the plant inhibited HIV-1 and HIV-2 replication, but more work would need to be done to further evaluate and utilize the plant against this virus (5).

Currently there are antiviral medications available to treat symptoms of HSV2 and reduce the frequency and severity of the symptoms when they present including acyclovir, famciclovir, and valacyclovir (47). There is a push for vaccine development to aid in prevention of initial infection of HSV2, as well as research into a topical microbicide that would prevent infection when topically applied before possible exposure (32). Research into the possibility of antiviral activity present in *Alchornea cordifolia* (Pennah) against HSV2 could uncover the potential for utilization of this plant extract as a treatment or preventative option against HSV2 infection.

Chapter 2

Literature Review

A majority of the human population (close to 100%) are infected or have been infected with a herpesvirus, a testimony to the widespread impact of this family of viruses (18). Herpesviruses got their name from the Greek herpein meaning “to creep” due to their ability to lay latent in the infected individual (34). This characteristic is what they are known for: establishing latency in an individual after initial infection, resulting in a lifelong recurrent viral shedding and possible recurrence of symptoms (23, 34). HSV2 is one of the three neurotropic herpesviruses that infect humans, others being herpes simplex virus type 1 (HSV1) and varicella zoster virus (VZV) (40). Resulting symptoms can include painful blistering and open sores, while some individuals do not show symptoms, but still undergo viral shedding as reported by the World Health Organization (47). The virus can be brought out of latency due to stimuli or sporadically (33). The latency of these viruses can result in severe, sometimes fatal, conditions for an individual in the future including shingles (from VZV), encephalitis and corneal blindness (from HSV1), and meningitis (from HSV2) (7, 40). Immunocompromised individuals (such as HIV sufferers) are more susceptible to recurrence and serious symptoms due to decreased immune response and ability (7, 47). The prevalence and ease of spread, especially due to asymptomatic carriers with established latency, and possibility of reemergence and cause of serious conditions, makes this virus group of interest in the medical community (1, 18, 7, 40, 47).

Properties of herpesviruses

Herpesviruses are nuclear replicating and are characterized as large enveloped double stranded DNA (dsDNA) viruses that can establish latency proving to be lifelong infections (7, 23, 40, 44, 47). Over one hundred different species of Herpesviridae have been uncovered, and of them there are eight herpes viruses that have been identified to infect humans, three of which are neurotropic (2, 23, 40). Across all viruses included in the Herpesviridae family there is a similar replication cycle, common structure, and the ability to establish latency, which includes periodic reactivation and viral shedding (23). Though all herpesviruses are common in those ways, they vary in others including cells they infect and remain latent and gene content resulting in three subfamilies: alpha, beta, and gamma (35). Herpesviruses are large being about 100-200 nm in size, icosahedral capsid shape, and considered complex regarding human viruses (18, 34). The dsDNA contains about 125-260 kbp which then encode roughly 70-200 proteins (18, 34). The basic structure includes a core containing the dsDNA inside an icosahedral protein capsid wrapped in another protein layer called the tegument encased by a final lipid envelope (16, 34). Throughout the many herpesviruses there are different sites of primary infection resulting in a range of symptoms depending on the specific virus within the family (7). Herpesviruses are host specific and have adapted to infect the host and continue to remain until end of life (34).

Herpes simplex virus type 2 belongs to the subfamily Alphaherpesviridae (35). They are known to have a short replication cycle that is completed in hours and establish latency in the sensory nerve ganglia in the host, specifically the dorsal root ganglia for HSV2 infections (40, 45). While neurons are important cells for establishing latency, epithelial cells also play a key role concerning infection and spread through mucosa (18). There are four main components making up the structure of a HSV2 virion: the core contains linear dsDNA, the icosahedral

protein capsid, the tegument, and the lipid envelope containing viral glycoproteins (34, 35, 38, 39, 45).

Herpes simplex type 2 Replication Cycle

The replication cycle of HSV2 is considered short in respect to other herpesviruses and is characterized as lytic and latent (7, 46). As an overview HSV2 goes through a typical viral replication cycle that includes binding/attachment to a susceptible host cell, penetration, uncoating, replication, assembly, and release of mature virion (12, 14).

1. Attachment

Viral attachment occurs when HSV2 comes in contact with a susceptible tissue having the receptors necessary for the glycoproteins on the envelope of the virion to bind (45). There are twelve glycoproteins (g) on the viral surface: gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM, and gN (45). The glycoproteins known to be involved in the attachment process are gB and gC, which allow the virion to bind to the heparin-sulfated proteoglycans (HSPGs) located on the cell surface (2, 43, 45). The initial bond with the cell surface receptors (HSPGs) is with gC, but in virions with a gC deficit gB can take over the roll resulting in decreased binding (1, 2, 43). Following the attachment to the HSPGs, gD binds to the other cellular receptors to strengthen the bond (43). The cellular receptors binding to gD that have been identified are: HveA/HVEM (herpes virus mediator) which is a part of the tumor necrosis factor receptor family (TNFR), Nectin1, and 3-O sulfated heparan sulfate, a modified heparin sulfated proteoglycan (2, 13, 43, 45). After the initial attachment facilitated by gC and gB, gD will interact with its receptor and initiate a conformational change resulting in a complex of gB and gH/gL to function in the fusion of the cellular and viral membranes (1, 43, 45).

2. *Penetration*

The entry process, or penetration, for HSV2 following attachment takes the form of viral fusion since HSV2 has a lipid envelope that can fuse with the cellular lipid membrane (1). The fusion of the viral lipid envelope and the cellular membrane is triggered by the binding of gD with one of the co-receptors, HveA/HVEM, Nectin-1, or 3-O sulfated heparan sulfate that causes the formation of a complex including gB, gH and gL allowing for fusion of the membranes and viral entry (1, 2, 43). The formation of the complex due to the gD binding results in the fusion of the membranes and eventual release of the capsid and tegument into the cytoplasm of the cell (43, 45).

3. *Uncoating*

Viral uncoating can occur once the capsid is transported to the nucleus by associating with microtubules (43, 45). While much of the tegument is disassociated (exception including VP1/2) to allow for docking at the nuclear pore complex (NPC) where the dsDNA is inserted into the nucleus in the nucleoplasm (14, 43, 45). The release of the viral linear dsDNA into the nucleus is termed uncoating (14).

4. *Replication*

Once the viral capsid is inside the nucleus, accompanied by the tegument protein VP16, it will begin the process of viral replication (29, 43). The viral protein VP16 acts as a transcription activator, or transactivator, by promoting the transcription and regulation of the viral immediate-early (IE) genes by recruiting transcriptional factors (9, 29, 45). There are three types of genes transcribed and translated by the host cellular machinery grouped by the order of viral protein expression (21, 36, 45). Once the viral proteins are transcribed, they exit the nucleus and enter the cytoplasm where they are translated, then reenter the nucleus to function in the viral

replication (36). The first wave of proteins transcribed are called the immediate early genes termed alpha, which can be transcribed due to host encoded factors and play a role in reducing host antiviral reactions and promoting the transcription of the next wave of viral proteins (21, 36, 43). Included in the alpha viral proteins are Infected Cell Proteins (ICPs) 0, 4, 22, 27, and 47 (21, 36). The next wave of viral proteins transcribed are the early genes, which are given the grouping beta (43). The beta genes promote the replication of the viral genome (43). The last wave of expression is termed the late genes, or gamma, which are mostly structural proteins that will comprise the mature viral particle therefore some do not reenter the nucleus but instead insert into the membrane of the rough endoplasmic reticulum and are glycosylated (21, 36, 43). Alpha genes reach their peak production at around 2-4 hours post infection, beta genes reach their peak production at 6-12 post infection, and gamma genes reach peak production at around 10-16 hours post infection (21).

Once the viral capsid enters the nucleus and the genome is released the linear dsDNA undergoes circularization necessary to produce progeny virions because the dsDNA does not elicit the double strand break response needed to replicate the DNA (31). This results in “rolling circle” replication of the circular concatemeric viral DNA that results in the linear copies of viral genome ready used in the packaging of new virions (21, 31, 43, 44, 45).

5. Assembly, Maturation, and Release

Assembly of the viral particle starts within the nucleus with the assembling of the capsid (21). The capsid is first assembled without containing the viral DNA, which is then (with the help of viral proteins) packaged inside the empty assembled capsid (21, 45). This can result in some capsids not getting packaged with DNA making them non-infectious (21). It is cited that the capsid then becomes enveloped in the inner nuclear membrane and then become de-

enveloped at the outer nuclear membrane as a way of exiting the nucleus as an assembled capsid into the cytoplasm (22, 27, 43, 45). The capsid acquires the tegument proteins in the nucleus and in the cytoplasm (22, 43). The capsid that had been enveloped in the inner nuclear membrane, then de-enveloped by the outer nuclear membrane, is now enveloped a second time in the trans-Golgi network (TGN) membranes through budding (21, 22). The trans-Golgi network membranes contain glycoproteins that are derived from glycoproteins translated into the endoplasmic reticulum during replication (22, 43). The now enveloped mature virion is transported to the cell surface by the TGN vesicle that fuses with the cellular membrane, releasing the enveloped mature virion (26).

Current Treatments

The high prevalence of herpes simplex viruses, like HSV2, calls for varying treatment options. This widespread infection remains latent causing the re-emergence of symptoms in many individuals. Antiviral drugs have been made available for the treatment of these symptoms and in some uses to suppress the flares, but none have been found to cure the infection (47). Antiviral drugs available for treatment of HSV2 include acyclovir, penciclovir, valaciclovir, and famciclovir, all of which are considered effective and can be utilized to alleviate symptoms and suppress episodic reemergence of HSV2 (3, 10, 37, 47). Though these antiviral drugs have proven effective at shortening the duration of symptoms and suppressing recurrent episodes they do not cure the infection therefore do not extinguish chance of transmission, and some resistance to the antiviral drugs has been noted rendering the antiviral drugs less than totally effective (10, 19). Each drug targeting HSV2 as an antiviral is showing action against DNA polymerase (41). There has been notable emergence of drug resistant HSV2 against acyclovir seen especially in

immunocompromised individuals taking this drug long term and regularly (41). There is continued support into further investigation of naturally occurring substances that prove effective as an antiviral, specifically against HSV (3).

Ethnomedicine has been practiced throughout the ages showing success in utilizing natural substances against various infections and diseases (17). Plants have been used to treat bacterial, fungal, viral, and plasmodial ailments by trial and error for the people utilizing the ethnomedicinal strategies (8, 17, 30). Naturally occurring substances proving to have antiviral properties have been identified through the exploration of ethnomedicinally used plants (30). Some examples of plants demonstrating antiviral activity are: *Geranium sanguineum* L. (against influenza virus), *Trichilia glabra* L. (against vesicular stomatitis virus), and *Boehmeria nivea* L. (against hepatitis B) (20, 30). There has also been research into the possibility of antiviral activity in *Carissa edulis* against HSV1 and HSV2 (30).

Alchornea cordifolia (Pennah) has long standing been a useful plant in regard to ethnomedicine showing great use as an antibacterial, antiplasmodial, and antifungal (8). Though there has been limited research done into its antiviral capabilities, *Alchornea cordifolia* has also shown promise as a potential antiviral in studies such as one done demonstrating antiviral activity against HIV-1 and HIV-2 in inhibiting viral replication (5). Another use of Pennah as an antiviral was investigated showing antiviral activity against respiratory syncytial virus (15). The spanning use of Pennah as an antimicrobial as well as some investigation into antiviral potential coupled with the need for more and naturally occurring treatment options against HSV2 appeals to the exploration of Pennah as a possible antiviral for HSV2 in our laboratory.

Chapter 3

Materials and Methods

Preparation of Plant Extract

Crude *Alchornea cordifolia* (Pennah) is obtained from the Congo. The plant material that was previously dried and stored was loaded into a cellulose extraction thimble. Soxhlet extraction was done using chemicals in sequence of organic solvent (a mixture of Methylene Dichloride (DCM), and Hexanes 1:1), followed by methanol, then water. Soxhlet extraction is used with a methanol solvent (1000 mL) to obtain the methanol fraction (MeOH) from the crude Pennah extract, which is dried in a drying oven and then collected in a scintillation vial.

Chromatographic Extraction on C-18 column

C18 column chromatography was used to further separate the Pennah MeOH extract using a methanol step gradient. First, Pennah MeOH extract (200 mg) was dissolved in 25 mL of dH₂O. The extract that did not go into solution was then dissolved using 15 mL of 100% methanol. Once equilibrated, the C18 was then washed with 100% methanol, then dH₂O. The extract dissolved in methanol was then washed through the C18 three times and collected. Then 30 mL of dH₂O, 10% methanol, 20% methanol, 30% methanol, and 100% methanol were run through the column, in that respective order. After each was washed it was collected resulting in collections from each was labeled according to the solution used for the collection. Each elution was then dried in a drying oven, scraped, and collected individually.

Thin Layer Chromatography

Thin-layer chromatography (TLC) was employed using the extract to visualize how many components may be apparent in the sample. The extract was dissolved in either dH₂O or MeOH, then centrifuged. The supernatant was collected and used for spotting on the silica plate in 5 μ L increments. The chamber was equilibrated with a wick, then the dried spotted silica plate was placed in the chamber with the solvent and left until the solvent reached the designated cut off point near the top of the silica plate. After running in the solvent, the plate was dried on a hot plate. The plates were viewed under long wave length ultraviolet light (365 nm) and short wavelength ultraviolet light (254 nm) to visualize bands.

Solvents used were:

Ethyl acetate : dH₂O : formic acid : glacial acetic acid (85:8:4:3)

Ethyl acetate : formic acid : glacial acetic acid : dH₂O (100:11:11:27)

Dichloromethane : methanol (50:50)

Testing for Chemical Class of Compound Present in Extract

Alkaloids were tested for in the Pennah MeOH extract is by preparing Dragendorff's Reagent and using 25 μ L combined with 0.5 mL of dH₂O and 25 μ L of Pennah MeOH extract.

An orange precipitate indicates the presence of alkaloids.

Phenols are tested for by adding 25 μ L of Pennah MeOH extract to 0.5 mL of Ferric chloride solution. Blue/green coloration indicates the presence of phenols.

Flavonoids are tested for using the Shinoda test, and pink or red coloration indicates presence of flavonoids.

Terpenoids are tested for using the Leibermann-Burchart test, and a green or green blue coloration or precipitate indicates presence of terpenoids.

Cell Culture

The Vero cell line (African green monkey kidney) was propagated and maintained with Dulbecco's Modified Eagle Media (DMEM) with added antibiotic/antimycotic, non-essential amino acids, sodium pyruvate, and 10% Fetal Bovine Serum (FBS). They were incubated in 37°C in 5% CO₂ until confluent monolayers formed. Once the cells formed a confluent monolayer on the cell culture flask, the cells were washed with phosphate buffer saline solution containing EDTA. The cells were then incubated in trypsin until the cells released from the flask. The mixture of cells and trypsin collected, centrifuged, resuspended in DMEM, and redistributed. The number of cell passages is limited, observed when the cells display morphological and/or physiological abnormalities.

Virus

Herpes Simplex Virus Type 2 (HSV2) was propagated in Vero cells. The stocks were stored at -70°C until use. In use virus was diluted to 100-150 plaque forming units (pfu) per milliliter (mL).

Plaque Assay

Viral assays were done using plaques to obtain results that quantified and determined the presence of antiviral activity. Vero cells were grown in 25 cm² culture flasks (t-25) to confluent monolayers. Confluent monolayered flasks were important for viral plaque assays because

plaques form from the infection of one cell and the viral production then infecting subsequent surrounding cells due to close proximity and higher probability of the viral particle coming in contact with the closer cells first to infect. The virus stored in -70°C is thawed and diluted to a working dilution of 100-150 pfu with DMEM without serum. The working dilution of virus is used to infect the confluent t25 flasks and incubated for 1 hour in 37°C . A negative control flask is infected with the same volume of DMEM without serum as virus was used in the positive and treated flasks. After incubation the virus is decanted and the flasks are washed with DMEM without serum to wash away unattached virus. Positive and negative control flasks were fed with DMEM with 10% FBS. Treated flasks were fed with varying concentrations of Pennah extract (such as $1000\mu\text{g/mL}$) dissolved in $20\mu\text{L}$ of DMSO and diluted with DMEM with 10% FBS. Flasks were incubated for 48-72 hours in 37°C .

This incubation time was used to allow for enough time and subsequent infections of surrounding cells to result in plaques that were visible by viewing with the eye after staining. The amount of time given allowed for enough viral cycles and subsequent infections in surrounding cells that gave a clear visible plaque, but not plaques that were too large and indistinguishable from each other. After 48-72 hours of incubation the flasks were observed individually for signs of cytopathic effects (CPE) and potential plaque formation. Plaques were counted and recorded for each flask. The plaques were able to be counted and recorded because a plaque was formed and considered to be an area on the monolayer that has the absence of cells. Therefore, after the crystal violet stains the remaining living Vero cells purple, the areas with no cells due to viral infection and cell death left behind a clear area on the flask. These areas lacking cells were formed in a circular pattern due to the infection of one cell and viral production (which results in cell death) causing the infection of surrounding cells due proximity resulting in

the subsequent viral infection. This repeated until the end of the incubation period resulting in circular areas lacking cells due to the death. These areas considered plaques were then counted and compared. The negative should have none due to no viral presence, the positive controls then were compared to the experimental groups determining antiviral activity in the extract, or lack thereof. A positive control had only virus infection without application of extract, while the experimental groups had extract applied. The amount of plaques formed on each was compared as a way to qualitatively say plaque reduction (less plaques in extract treated groups) was or was not occurring, and by counting and calculation was a way to say how effective the extract was as an antiviral agent.

Staining Protocol

After the 48-72-hour incubation period and observable viral plaque formation in positive controls the cells the flasks were decanted into a waste jar containing disinfectant. The flasks of cells were then fixed using 2.5% formaldehyde solution for 10 minutes, then stained with crystal violet (0.1% in 35% methanol) for 10 minutes. The flasks were rinsed three times with water then dried in the incubator. The observable plaques were then counted in each flask. The positive and experimental groups were compared using the below calculation for plaque reduction (R%):

$$R\% = [(P_{pc} - P_e) / P_{pc}] \times 100$$

R%: percent plaque reduction

P_{pc}: plaque count in positive control

P_e: plaque count in experimental flask

Post-Adsorption Assay

The post-adsorption, or post-attachment, assay was performed to determine if the extract showed antiviral activity against the virus by comparing the number of plaques in the positive controls to how many plaques developed in the treatment groups. Vero cells were grown in 25 cm² culture flasks (t-25) to confluent monolayers. The virus stored in -70°C is thawed and diluted to a working dilution of 100-150 pfu with DMEM without serum. The working dilution of virus is used to infect the confluent t25 flasks and incubated for 1 hour in 37°C. A negative control flask is infected with the same volume of DMEM without serum as virus was used in the positive and treated flasks. After incubation the virus is decanted and the flasks are washed with DMEM without serum to wash away unattached virus. Positive and negative control flasks were fed with DMEM with 10% FBS. Treated flasks were fed with varying concentrations of Pennah extract (such as 1000µg/mL) dissolved in 20µL of DMSO and diluted with DMEM with 10% FBS. Flasks were incubated for 48-72 hours in 37°C. After 48-72 hours of incubation the flasks were observed individually for signs of cytopathic effects (CPE) and potential plaque formation. Plaques were counted and recorded for each flask. The viral assay is stained using 2.5% formaldehyde solution to fix the cells, and 0.1% in 35% methanol crystal violet.

Post-Adsorption Assay with Modifications to Time Cells were Exposed to Extract

These assays are carried out as outline in the above section, with the exception of when the extract treatment is applied and removed from the cells after the 1 hour of incubation for viral attachment and wash. At certain time intervals the extract was added and removed from different treatment flasks. This gave information regarding how the extract may have affected viral replication.

A version which this assay was performed included specific time intervals surrounding the addition of plant extract on infected cells. Following 1 hour of incubation of confluent t25 flasks of cells infected with 100-150 pfu of virus the flasks were washed. Each flask was fed with DMEM with 10% FBS until a specified time interval. At this point the flasks were fed with plant extract in DMEM with 10% FBS and incubated for 48 hours in 37°C. At the end of the incubation time flasks were observed for CPE and plaque formation, fixed, stained, and plaques counted.

Another version of this assay was performed with the removal time interval of the plant extract modified. Confluent cells were infected with 100-150 pfu for 1 hour in 37°C then washed with DMEM without serum. The flasks were then fed with extract diluted with DMEM with 10% FBS. At the end of the indicated time interval the flasks were washed with DMEM without serum and fed with DMEM with 10% FBS until the end of the 48 hour incubation in 37°C. At the end of the incubation time flasks were observed for CPE and plaque formation, fixed, stained, and plaques counted.

Neutralization Assay

In the neutralization assay the virus was combined with the plant extract before infection of the cells to determine if the plant extract neutralizes the virus. The plant extract was dissolved in 20 µL of DMSO and brought to desired concentration using DMEM with 10% FBS. Virus diluted to 100-150 pfu with DMEM without serum was combined with the plant extract and incubated for 20 minutes at 37°C. Following the 20 minutes of incubation the mixture of plant extract and virus were used to infect confluent monolayer t25 flasks of cells. Positive controls were infected with a mixture of virus and DMEM without serum that incubated for 20 minutes,

and negative controls were infected with DMEM without serum that incubated for 20 minutes. After infection, the flasks were incubated for 1 hour at 37°C then washed with DMEM without serum, and subsequently fed with DMEM with 10% FBS and incubated for 48-72 hours in 37°C. At the end of the incubation time flasks were observed for CPE and plaque formation, fixed, stained, and plaques counted.

Pre-infection Treatment Assay

In the pre-infection treatment assay the plant extract was applied to the Vero cells overnight before infection of the cells to determine if pre-exposure to the plant extract had any effect on the cells that may affect the resistance to HSV2 infection. The plant extract was dissolved in 20 µL of DMSO and brought to desired concentration using DMEM with 10% FBS. The plant extract was applied to the confluent t25 flasks of the experimental groups, and DMEM with 10% FBS was applied to the positive and negative control confluent t25 flasks. After incubating at 37°C overnight all t25s were decanted, washed with DMEM without serum, and infected with virus diluted to 150-200 pfu with DMEM without serum, with the exception of the negative control that was infected with DMEM without serum. After infection, the flasks were incubated for 1 hour at 37°C then washed with DMEM without serum, and subsequently fed with DMEM with 10% FBS and incubated for 48-72 hours in 37°C. At the end of the incubation time flasks were observed for CPE and plaque formation, fixed, stained, and plaques counted.

Indirect Immunofluorescence Assays

Indirect immunofluorescence assays were performed to visualize the virus in the cells. The method utilized two sets of antibodies, a primary and a secondary. The primary antibody

attached to the viral antigen, with a secondary antibody following and attaching to the primary antibody. Following treatment with the primary and secondary antibodies the complex created can be observed under the fluorescence microscope. This method for visualization was used for observing viral production in extract treated cells compared to positive controls.

Cells at semi-confluency (around 50-60%) were used for indirect immunofluorescence assays to aid in visualization. Cells were washed twice with DMEM without serum that was kept on ice prior and for the duration use in this setting. When using this method to visualize viral replication at certain time intervals in extract treated versus positive control (no plant extract) treated cells the cells were infected with 100-150 pfu of virus and incubated for 1 hour in 37°C. Following the incubation, the cells were washed with the DMEM without serum and fed with DMEM with 10% FBS. This counted as zero hours and at determined time intervals following the cells were fixed and treated with primary and secondary antibody. When using this method to observe attachment of the virus in extract treated cells compared to positive controls more virus was required so about 100,000-150,000 pfu was used. This high level pfu of virus was pre-exposed to plant extract on ice for 20 minutes before infection of the cells. The cells were kept on ice and infected with the ice-cold mixture of extract and virus for 2 hours. After the 2 hours of incubation of the virus and extract mixture (virus and DMEM without serum for the positive control) on ice, the cells were washed three times with ice cold DMEM without serum. Immediately following being washed with SFM three times the cells were fixed and treated with primary and secondary antibody.

After cells were washed three times with cold DMEM without serum in either situation above, the cells were fixed with 5 mL of 2% formaldehyde. All cells in the experiment set were treated together. The formaldehyde is then decanted and 2-3 mL of DMEM without serum is

added to the flasks of cells and placed on the shaker for 5 minutes. Following the 5 minutes the cells are washed again twice more with 2-3 mL of DMEM without serum. This is the washing process used moving forward in this method. In cases of the first use of the method to determine viral production at certain time intervals the cells were then permeabilized with 5 mL of 100% methanol. This was decanted and the cells were washed using the shaker and subsequent 2 wash protocol. In the case of visualizing viral attachment kept under full cold conditions the permeabilization step is not used to help focus on just the attachment step of the viral cycle. Next, 3-4 mL of primary antibody (rabbit anti-HSV2) was applied to the cells and placed on the shaker for even distribution for 1 hour. Following, the cells were washed three times as described prior. About 3-4 mL of secondary antibody (goat anti-rabbit conjugated with 546 nm AlexaFluor) is then applied to the cells and left on for 1 hour on the shaker. Subsequently washed using the three-wash method and kept in 3 mL of DMEM without serum and refrigerated until viewed under the fluorescence microscope (Olympus IX71 inverted immunofluorescent microscope excited at 546 nm).

Chapter 4

Results

Research on the antiviral activity of African traditional medicinal plants was conducted on crude and column fractionated extracts of *Alchornea cordifolia* (Pennah) against Herpes Simplex Virus Type 2 (HSV2) in Vero cells. The methanol extract was further fractionated using a C-18 silica in a flash column chromatography in an attempt to isolate the active compound. First, we had to establish what the major plant compounds that could be detected in the extracts.

Chemical Class of Compound Present in Extract

Chemical analyses of the plant extract were performed to determine the possible presence of alkaloids, flavonoids, terpenoids, and phenols as described in the Materials and Methods section (Chapter 3).

Crude Pennah MeOH Extract and 30% Pennah MeOH Fraction

Tested positive for the presence of alkaloids, phenols, and terpenoids and negative for the presence of flavonoids.

Thin-Layer Chromatography

Thin-layer chromatography was utilized as a way to visualize potential number of major components in the plant extract, and in fractions that exhibited antiviral activity fractions.

Preliminary TLC results are in Figure 1.

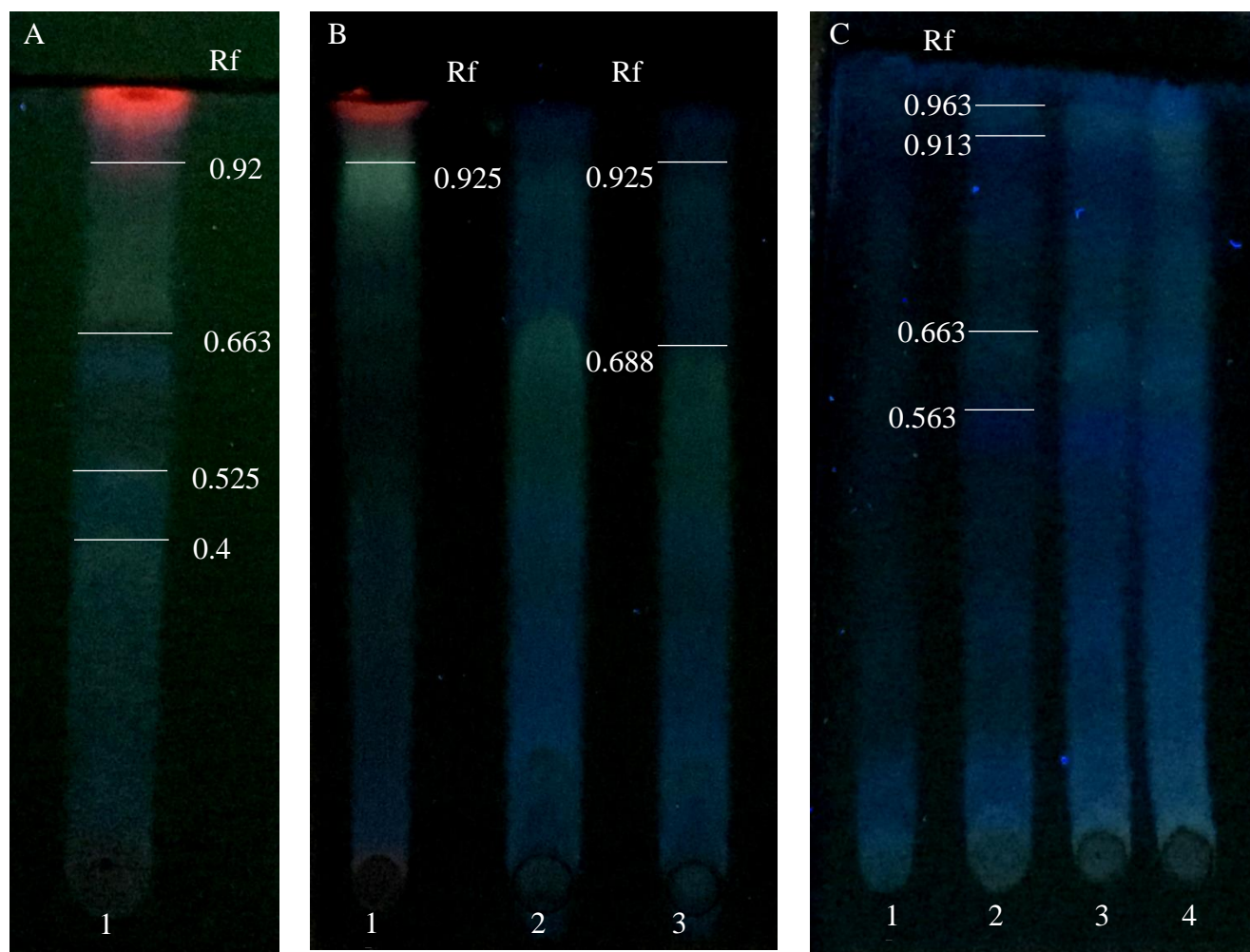


Fig. 1: Displayed in A, B and C are images of TLC plates under the long wavelength UV light (365 nm). The TLC plates were run with the solvent ethyl acetate : dH₂O : formic acid : glacial acetic acid (85:8:4:3). Image A shows the crude Pennah MeOH extract (Lane 1A) with four distinct bands and the Rf values. Image B shows a comparison of the crude Pennah MeOH (Lane 1B) run alongside 20% Pennah MeOH (Lane 2B) and 30% Pennah MeOH (Lane 3B). Lane 1B and 3B are being compared with a band at Rf value 0.925 appearing in both lanes. Image C depicts four lanes of increasing concentrations of 30% Pennah MeOH after being run twice in the solvent to further resolve the possibility of more bands. Lane 2C and Lane 3C show the bands the most resolved and bright enough to distinguish. 3B compared to 2C shows two bands at Rf values 0.963 and 0.913 resolved from the singular band at Rf value 0.925 following the second run in the solvent.

Detection for Antiviral Activity

1. Post-Adsorption Assay

Post-adsorption, or post-attachment, assays were performed to determine if the plant exhibited antiviral activity after the Vero cells were exposed to HSV2 and time for attachment of the virus was allowed (1 hour) before treatment with plant extract was applied to the cells.

Preliminary results of Crude Pennah (water extract and MeOH extract) in Table 1.

1a. Crude Pennah MeOH Extract

Crude Pennah MeOH extract was applied to the cells after the initial infection to determine if the plant extract treatment would result in antiviral activity against HSV2. Results of this assay are displayed in Table 2.

1b. Antiviral Activity in Crude Extract run on C-18 Colum eluted with MeOH

Results of the experiments done to determine the plaque reduction potential of extract fractionated on C-18 column with increasing concentration of MeOH are shown in Table 3 and in Figure 2.

1c. 30% MeOH Pennah MeOH Extract

Experiments were done to determine the plaque reduction potential of 30% Pennah MeOH using the post-adsorption assay. Results of the plaque reduction percentages when using the 30% MeOH fraction of Pennah MeOH (referred to as 30% Pennah MeOH) is shown in Table 4.

Table 1: Antiviral Activity Screening in Crude Pennah		
$\mu\text{g/mL}$	Crude Pennah Water Plaque Reduction (%)	Crude Pennah MeOH Plaque Reduction (%)
1000	95	100
500	81	100
250	65	100
125	49	70

Table 1: Antiviral activity as plaque reduction percentages after a post-adsorption screening of crude Pennah water extract and crude Pennah methanol extract against HSV2. Data shows antiviral activity in both crude extracts. Higher plaque reduction and more effective antiviral activity is observed in crude Pennah MeOH extract in comparison to the water extract.

Table 2: Antiviral Activity in Crude Pennah MeOH	
$\mu\text{g/mL}$	% Plaque Reduction
1000	100
500	100
250	100
125	63
62.5	42

Table 2: Resulting plaque reduction percentages displaying antiviral activity seen in crude Pennah MeOH against HSV2. At a concentration of 1000 $\mu\text{g/mL}$ showed mild toxicity to the Vero cells, and at 500 $\mu\text{g/mL}$ very slight toxicity to the cells. Concentrations under 500 $\mu\text{g/mL}$ did not show toxic effects on the cells.

Table 3: Antiviral Activity in the Collected C18 Fractions					
Fraction	dH ₂ O	10% MeOH	20% MeOH	30% MeOH	100% MeOH
% Plaque Reduction	84	85.8	100	100	29.6

Table 3: Results of post-adsorption screening with collected C18 fractions against HSV2 for determination of fraction with most antiviral activity potential to move forward with experimentally. Results of a screening done with 62.5 $\mu\text{g/mL}$ at low pfu. Similar results indicated for 20% MeOH and 30% MeOH fractions necessitates further experimental evaluation at lower concentrations.

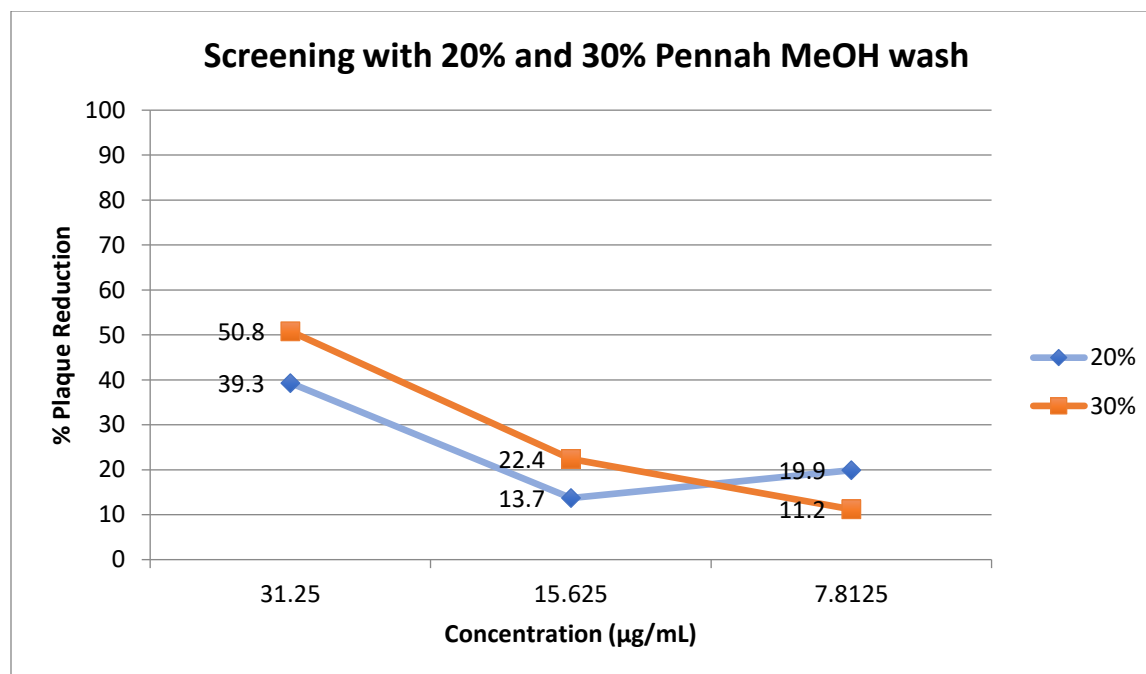


Fig. 2: This line graph displays the data collected during a post-adsorption screening against HSV2 in Vero cells of 20% Pennah MeOH fraction and 30% Pennah MeOH fraction to determine which fraction would be used moving forward.

Table 4: Antiviral Activity of 30% Pennah MeOH Extract	
µg/mL	% Plaque Reduction
1000	100
500	100
250	97.8
125	64.5
62.5	48
30.25	50.8
15.625	22.5
7.8125	11.2

Table 4: Results of post-adsorption assay in the form of plaque reduction percentages of 30% Pennah MeOH against HSV2 in Vero cells. Demonstrates the antiviral activity potential of this fraction of Crude Pennah MeOH.

2. Neutralization of the infectivity of the HSV2 by 30% Pennah MeOH Fraction

Neutralization assays were performed to determine if viral exposure to the plant extract before infection would result in the neutralization of the infectivity of the HSV2 before application to the Vero cells. This type of assay was performed by combining HSV2 with plant extract and incubation for 20 minutes before using the mixture to infect the Vero cells.

Results showed that 125 µg/mL of 30% Pennah MeOH extract neutralized 61.7% of HSV2. This data is referenced in Figure 5.

3. Pre-infection Treatment Assay

A pre-infection treatment assay was performed using 30% Pennah MeOH to determine if the extract, when applied to the cells overnight before infection, would prove to be affective against HSV2 in the form of plaque reduction compared to positive controls. This assay was performed using concentrations of 125 µg/mL of 30% Pennah MeOH, and 250 µg/mL of 30% Pennah MeOH. Plaque reduction for 125 µg/mL was 28.8%. Plaque reduction for 250 µg/mL was 84%. Comparison of results of pre-infection treatment of cells with 30% Pennah MeOH and post-infection treatment of 30% Pennah MeOH can be found in Figure 3.

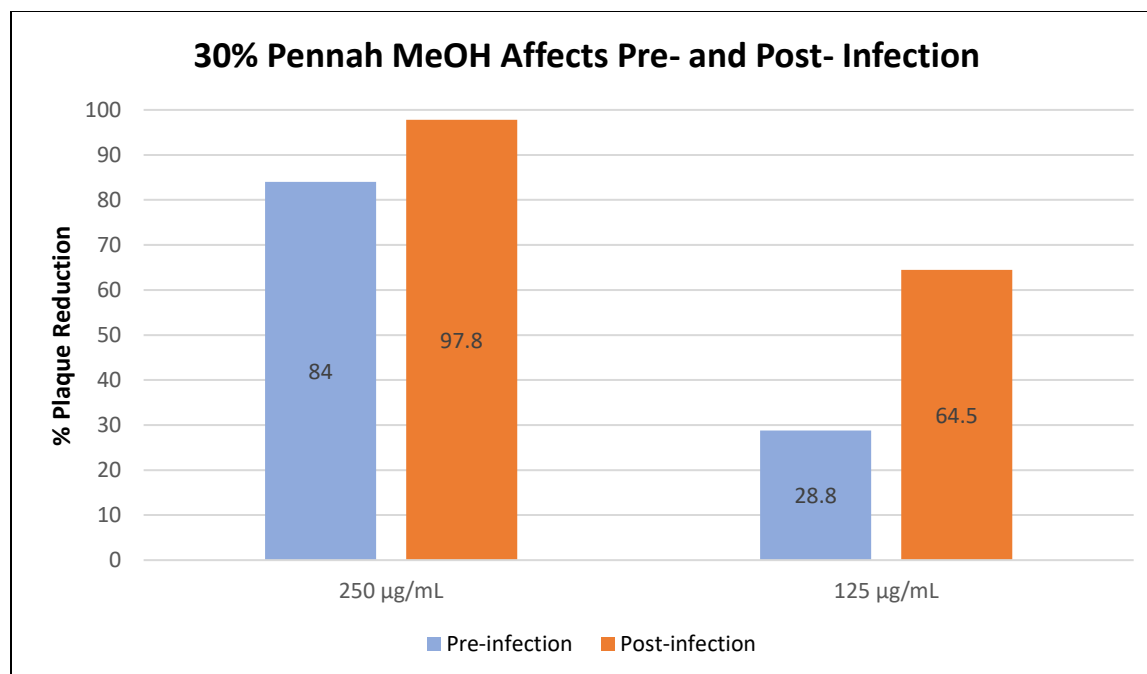


Fig. 3: The bar graph shows the comparison of percent plaque reduction seen pre-infection and post-infection against HSV2 in Vero cells of 30% Pennah MeOH plant extract. Pre-infection, which showed less antiviral activity, was when the cells were treated with 30% Pennah MeOH before infection with HSV2. Post-infection was when the Vero cells were infected with HSV2 and then treated with 30% Pennah MeOH following the infection which showed more antiviral activity.

4. Post-Adsorption Assay with Modifications to Time Cells were Exposed to Extract

Time sensitive assays were performed with changes occurring the addition of extract and the removal of extract after initial infection (1-hour post incubation of virus and Vero cells). The extract used in these experiments was the 30% MeOH fraction Pennah MeOH (referred to as 30% Pennah MeOH).

4a. Addition of Extract at Different Time Intervals Post-Infection

Vero cells were infected with HSV2 and incubated for 1 hour before being fed with DMEM with 10% FBS. Two hours after the infection some of t25 flasks were decanted and washed with SFM then 30% Pennah MeOH at a concentration of 125 µg/mL was applied and the flasks were incubated until the end of the 48 hour full incubation time. Eight hours after the infection another

group of the t25 flasks were decanted and washed with SFM then 30% Pennah MeOH at a concentration of 125 $\mu\text{g/mL}$ was applied and the flasks were incubated until the end of the 48 hour incubation time. The two groups of flasks with addition of plant extract at 2 or 8 hours was compared to the positive controls that were not treated with plant extract. The flasks with 125 $\mu\text{g/mL}$ 30% Pennah MeOH applied at 2 hours until end of incubation resulted in a plaque reduction of 54.5%. The flasks with 125 $\mu\text{g/mL}$ 30% Pennah MeOH applied at 8 hours until the end of incubation resulted in a plaque reduction of 57%. These values are displayed in Figure 4 and referenced in Figure 5.

4b. Removal of Extract at Different Time Intervals Post-Infection

Vero cells were infected with HSV2 and incubated for 1 hour before being treated with 125 $\mu\text{g/mL}$ 30% Pennah MeOH. Two hours after the application of the plant extract some of the t25 flasks with the extract applied were decanted and washed with SFM, fed with DMEM with 10% FBS, and incubated until the end of the 48 hour incubation. The remaining t25 flasks that were treated with plant extract were incubated until 8 hours after application of extract at which point the plant extract was decanted, the flasks were washed with SFM, and then fed with DMEM with 10% FBS and incubated until the end of the 48 hour incubation. The flasks that had the 125 $\mu\text{g/mL}$ 30% Pennah MeOH removed after 2 hours of treatment after initial infection resulted in a plaque reduction of 8.2%. The flasks with 125 $\mu\text{g/mL}$ 30% Pennah MeOH removed at 8 hours after application of extract resulted in a plaque reduction of 11.5%. These values are displayed in Figure 4 and referenced in Figure 5.

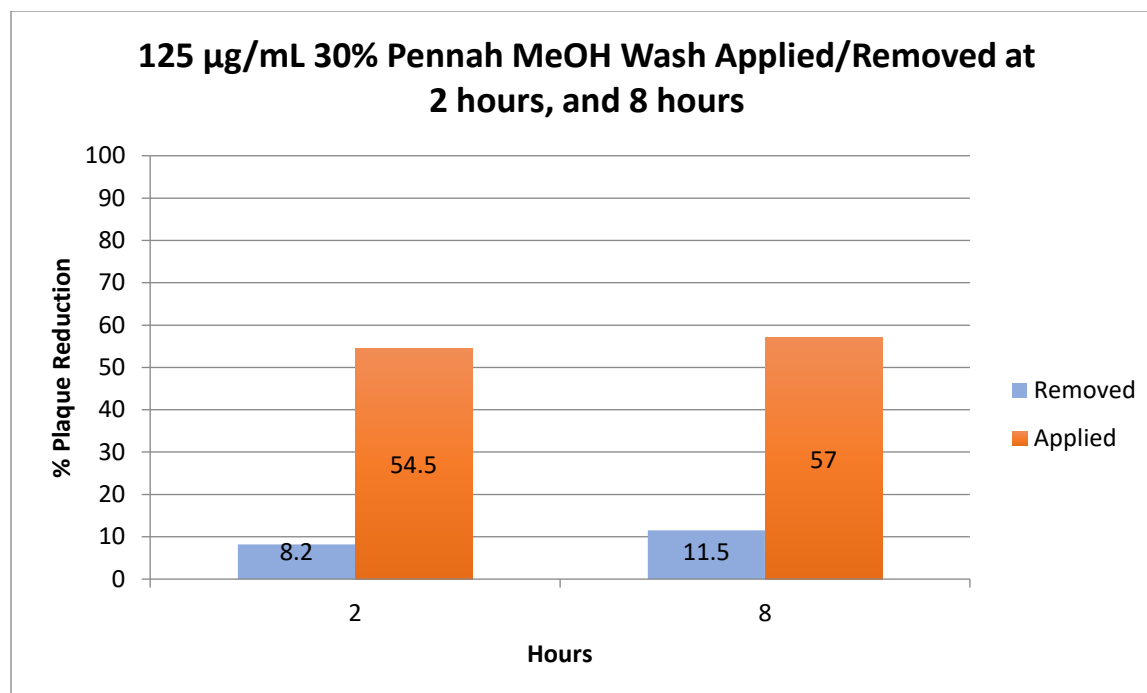


Fig. 4: Percent reduction seen when treatment of 125 μ g/mL of 30% Pennah MeOH wash was applied/removed at 2 hours, and 8 hours. This graph shows the difference in plaque reduction of the plant extract when utilized against HSV2 in Vero cells.

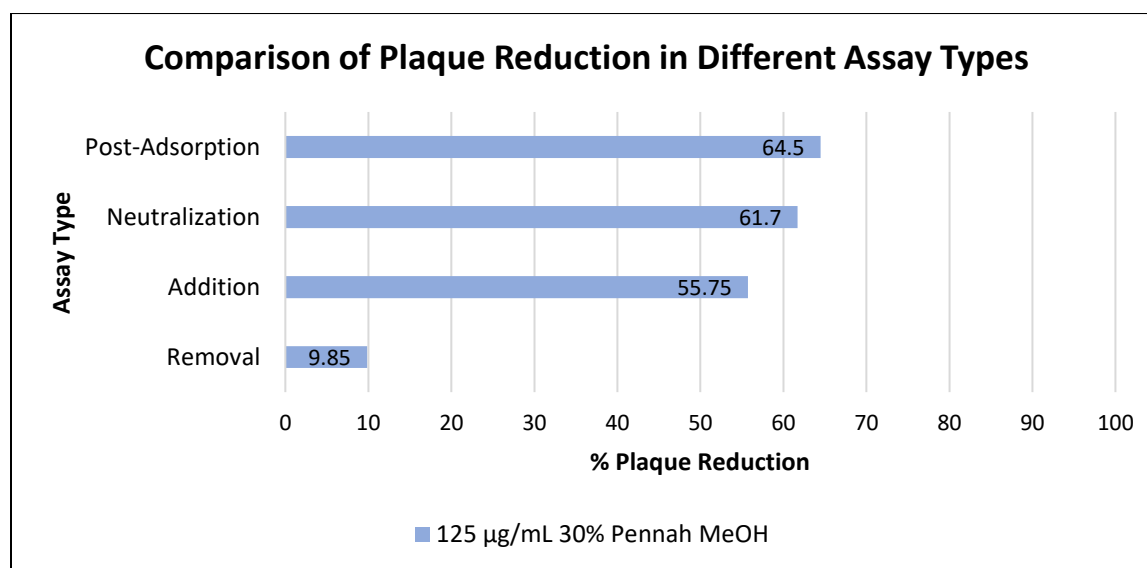


Fig. 5: The bar graph represents the plaque reduction seen when utilizing 125 μ g/mL 30% Pennah MeOH extract in varying types of plaque assays. Post-adsorption represents Vero cells treated with plant extract following infection of HSV2. The bar labeled neutralization is the resulting plaque reduction of cells infected with HSV2 combined with plant extract prior to infection with the combination. The addition bar represents the average plaque reduction observed after the addition of the plant extract at 2 and 8 hours. The removal bar represents the average plaque reduction observed after the removal of the plant extract at 2 and 8 hours.

5. Immunofluorescence Assays

Indirect immunofluorescence was the technique used to visualize HSV2 and its course of infection in Vero cells, and how that course would be affected by treatment of the plant extract. Positive control images were taken for reference in Vero cells that were infected with HSV2 and not treated with plant extract to visualize the progression of infection without treatment at certain time intervals of the viral infection.

5a. Immunofluorescence with Post-Adsorption Assay

Results in Figure 6 demonstrate the comparison of HSV2 infection in Vero cells and HSV2 infection of Vero cells that were treated with plant extract. Images in Column 2 (Images 2a-2c) show the resulting viral infection after 48 hours of incubation and after treatment with 125 $\mu\text{g/mL}$ of 30% Pennah MeOH. Column 1 (images 1a-1c) show the pattern of infection following the 48 hour incubation of HSV2 infected Vero cells with no treatment, considered positive controls. The resulting images show differences in the viral infection between the two groups. Both groups show cells infected with HSV2 because any visualization is due to the presence of the HSV2 virions. The cells in the positive control groups are shown to be in the later stages of infection and showing cytopathic effects of the cells balling up instead of being stretched out like the cells remained in the treatment groups as well as evidence of cell fusion resulting in multinucleated cells. Although the cells are infected in the treatment groups the virus at 48 hours post infection is not having the same balling effect on the cells. Another notable difference is where the abundance of virus is located in the infected cells at this point of infection. In images 2b and 2c the area of the cells that has brighter fluorescence indicating more virus is the nucleus, while in images 1a-1c of the positive controls it shows the cells observed in those groups were characterized by larger amount of virus in the cytoplasm. Although some cells in the treatment

group had more virus in the cytoplasm (1c) the virus was more centralized in small clusters instead of dispersive throughout the cytoplasm like in the positive controls and those cells did not show the fusion of cells resulting in multinucleated cells like it did in the positive controls with virus in the cytoplasm.

5b. Immunofluorescence with Post-Adsorption Assay with Time Modifications

Visualizing the results for this assay are found in Figure 7. All groups were fixed at 28 hours post infection with HSV2. Images 5a and 5b are positive control images that had no treatment with plant extract at any point in the infection and are used for baseline and comparison images. The positive control images 5a and 5b show a field of view with many infected cells, as well as balling of cells occurring and fusion of cells resulting in multinucleated cells. Those are all indicative of normal CPE caused by HSV2 in Vero cells. It can also be noted the higher presence of virus in the cytoplasm of the cells diffuse when the cells are still stretched, and pushed to the outside when the cells are shown balled up with less amount of cytoplasm visible due to decreased surface area because of the balling effect caused by HSV2. Rows 1-4 show cells treated with 30% Pennah MeOH (rows 1-2 with 125 $\mu\text{g/mL}$, rows 3-4 with 250 $\mu\text{g/mL}$) that were either added or removed at 2 or 8 hours. This was to reflect the resulting plaque reduction percentages seen as a result of the post-adsorption assay with time modifications. The cells treated with plant extract at 2 or 8 hours (1a-4a) have similar results and show less cells in the field of view infected with HSV2, as well as more virus in or around the nucleus than compared to the positive controls. This is reflective of the prior comparisons of the post-adsorption assay and is more characteristic of early stages of infection compared to the positive controls. In all 4 images 1a-4a there are less cells in the field of view infected with HSV2, and between 1a-2a and 3a-4a there are visibly less cells infected with HSV2 in the field of view in 3a-4a because it is

treated with a higher concentration of plant extract. This is demonstrating that the amount of plant extract used in experiments does have an impact on the antiviral activity and effectiveness of the plant on the viral replication of HSV2 in the Vero cells. This shows the extract at both 125 $\mu\text{g/mL}$ and 250 $\mu\text{g/mL}$ and both added at 2 or 8 hours is affecting the viral replication in the Vero cells. Images 1b-4b show cells with either 125 $\mu\text{g/mL}$ or 250 $\mu\text{g/mL}$ of 30% Pennah MeOH removed at 2 or 8 hours. Based on the plaque reduction percentage results of these groups from the assay performed it showed very little plaque reduction compared to the groups with extract added at these times. The plaque reduction percentage was between 8-12% when the plant extract was removed. These images are very similar to the positive controls in the number of cells infected in the field of view as well as some balling of cells and fusion of cells showing large multinucleated cells with viral presence mostly seen in the cytoplasm diffusely. Though all 4 images are similar to the positive controls it is noted that the least number of cells infected in a field of view is in image 4b: the highest concentration of extract in the experiment and left on the longest before removal at 8 hours compared to 2 hours. This is demonstrated by these images showing very similar infection pattern and number of cells in the field of view infected when compared to the positive control images 5a and 5b.

5c. Immunofluorescence with Neutralization Assay

HSV2 at a high pfu of 100,000-150,000 was combined under ice cold conditions with the plant extract. 1000 $\mu\text{g/mL}$ of 30% Pennah MeOH was used in equal quantities of virus combined on ice for 20 minutes before infection of flasks of Vero cells kept on ice for a total infection time of 2 hours. Positive controls were infected with equal parts ice cold SFM and high pfu HSV2 and infected with the combination under the same conditions for 2 hours. At this time the cells were

fixed and treated for indirect immunofluorescence with the absence of the permeabilization step with 100% MeOH to ensure focus on the attachment of virus to the cells. Figure 8 shows the resulting images to further understand if the plant extract was preventing attachment of the HSV2 to the cells, resulting in less infected cells and therefore plaque reduction, or if the HSV2 was still attaching and the plant extract was affecting another part of the viral replication cycle. Column 1 shows positive controls and Column 2 shows cells infected with virus combined with 30% Pennah MeOH. 1a and 1b showing the positive controls displays pinpoint areas of fluorescence indicating high viral areas on the cells. Images 2a and 2b demonstrating the treatment groups show similar amounts of pinpoint fluorescence areas demonstrating the presence of viral attachment in the treatment groups comparable to the same seen in the positive controls. Based on the images it is shown that the attachment of HSV2 still occurs in extract exposed virus, meaning the plant extract is not interfering with the viral attachment step of the infection cycle, but instead another step further along inside the infected cells.

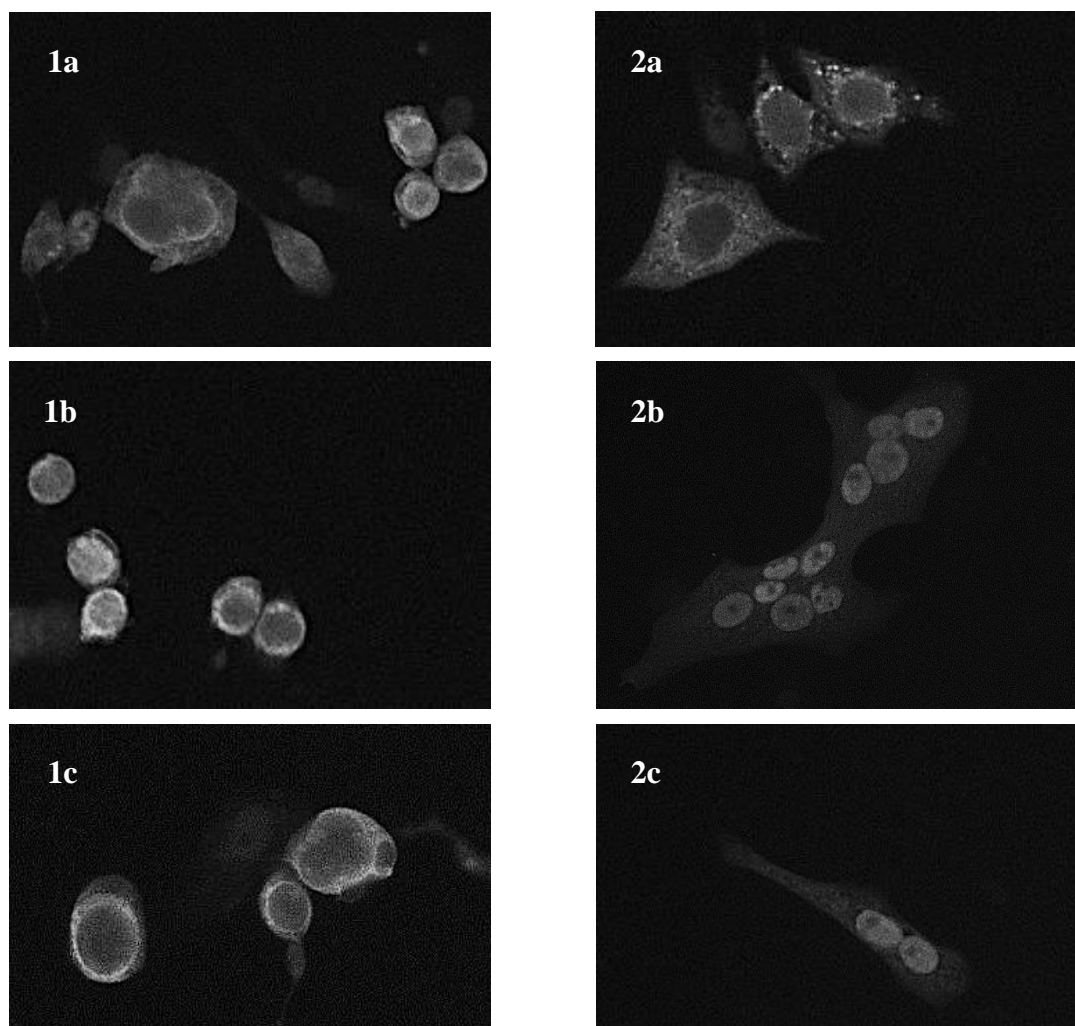


Fig. 6: The images above are micrographs of indirect immunofluorescence utilized on a post-adsorption assay. The images show Vero cells fixed at 48 hours post HSV2 infection. Column 1 (images 1a, 1b, and 1c) displays pictures of positive control cells, which were infected with HSV2 and not treated with plant extract. The positive control images show cells that are in later stages of infection and showing signs of cytopathic effects (CPE) CPE visible in these images are ballooning of cells, more viral presence diffuse in the cytoplasm instead of the nucleus of the cells, and fusion of cells resulting in multinucleated cells. Column 2 (images 2a, 2b, and 2c) contains images of cells infected with HSV2 and subsequently treated with 125 $\mu\text{g/mL}$ of 30% Pennah MeOH. In these three images at the same time of infection as the positive controls the cells are seen to be in earlier or less progressed infection stages. Most cells do not have the virus concentrated in the cytoplasm like the positive controls, and if they do (2a) they are not fused or multinucleated as well as showing more pinpointed areas of virus instead of diffuse in the cytoplasm. In cells exhibiting fusion and multinucleation (2b and 2c) the virus is not diffuse in the cytoplasm, but instead concentrated in the nucleus indicative of an earlier stage of infection where the virus has not exited the nucleus into the cytoplasm yet.

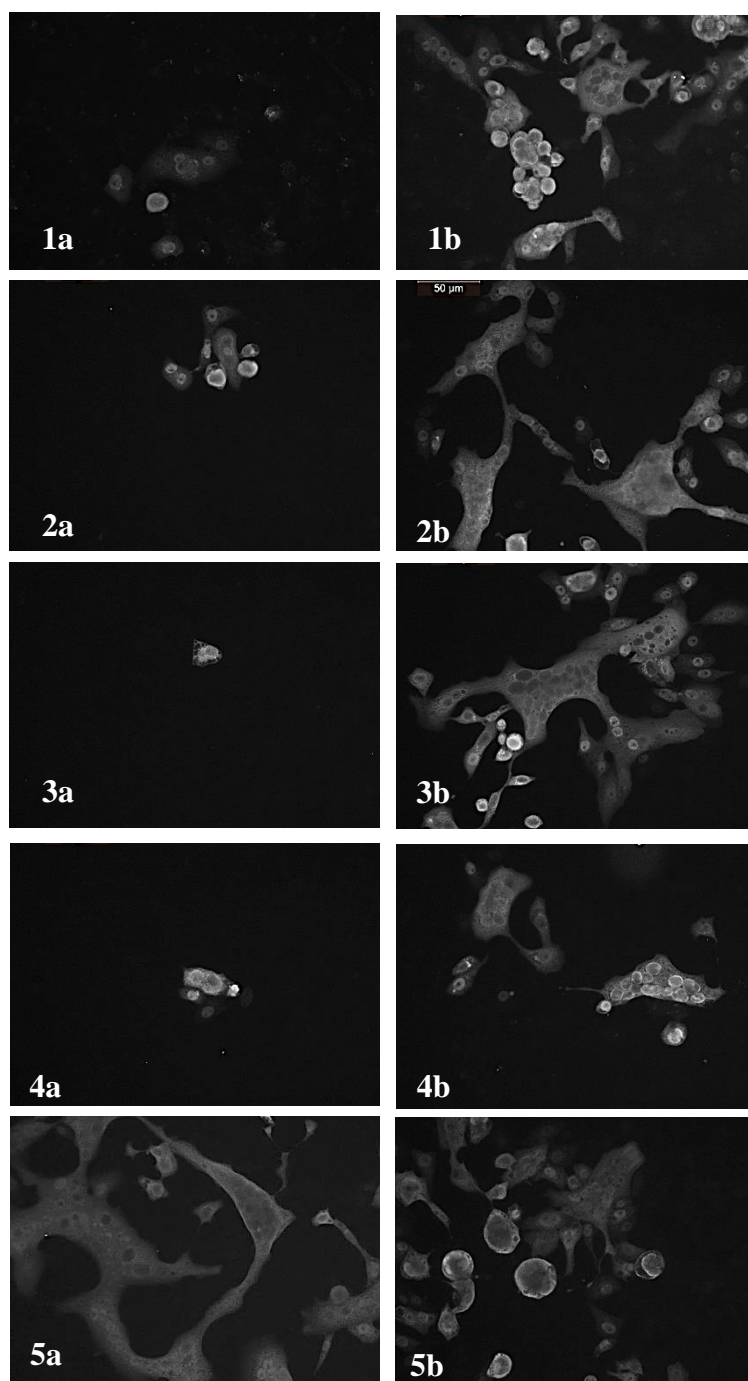


Fig. 7: Displayed are micrographs of indirect immunofluorescence utilized on a post-adsorption assay with modifications to time cells were exposed to extract. All images represent Vero cells fixed at 28 hours post HSV2 infection. Rows 1 and 2 represent cells treated with 125 µg/mL 30% Pennah MeOH. Rows 3 and 4 represent cells treated with 250 µg/mL 30% Pennah MeOH. Row 5 represents positive control cells that were infected, but not treated with any plant extract. 1a and 3a display cells with extract added at 2 hours post initial infection and left on for the 28 hours. 2a and 4a display cells with extract added at 8 hours post initial infection and left on for the 28 hours. 1b and 3b display cells with extract removed 2 hours post initial infection and subsequently fed with DMEM with 10% FBS for the 28 hours. 2b and 4b display cells with extract removed 8 hours post initial infection and subsequently fed with DMEM with 10% FBS for the 28 hours. Observe in the positive controls typical CPE of Vero cells infected with HSV2, such as balling and fusion resulting in multinucleated cells and more diffuse viral presence in the cytoplasm. In images 1a-4a showing the addition of extract there are much less cells in the field of view infected as well as presence of virus in the nucleus and concentrated virus around the nucleus instead of diffuse in the cytoplasm. Also note in images 1a-2a treated with lower concentration of plant extract more cells are infected showing the higher concentration of plant results in more antiviral activity. Note in images 1b-4b the cells are very similar to the positive controls with many cells in the field of view infected and balling of cells as well fusion resulting in large multinucleated cells like the positive controls.

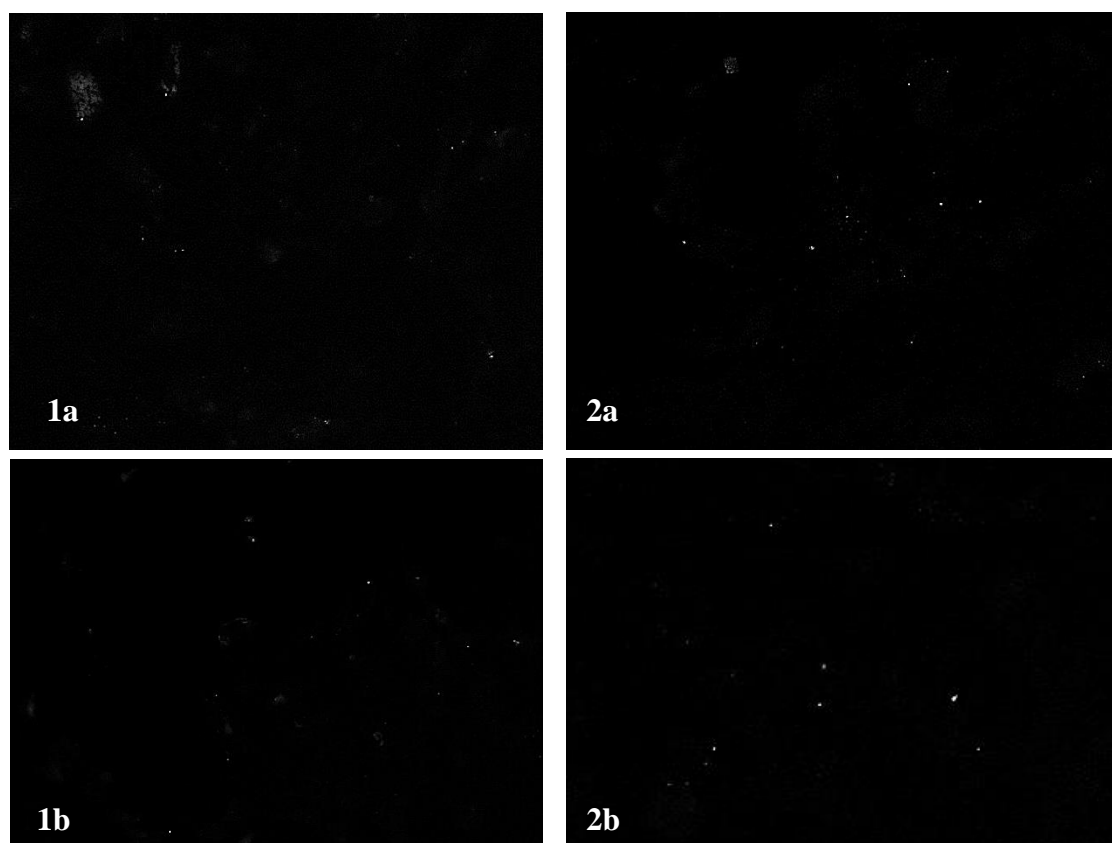


Fig. 8: Micrographs depicting indirect immunofluorescence utilized on a neutralization assay with a high pfu of 100,000-150,000 and a 2-hour incubation time on ice before Vero cells were fixed. Column 1 represents the positive control cells with no plant extract and virus combination. Column 2 represents cells infected with the virus in combination with 1000 $\mu\text{g/mL}$ 30% Pennah MeOH. In images 1a and 1b the positive controls pinpoints of fluorescence are indicative of viral attachment to the cells in the field of view. The time of exposure to HSV2 and under the ice-cold conditions it only allowed for attachment to occur without penetration to result in visualization of attachment of virus in cells and nothing further. The positive images showing the pinpoints compares to images 2a and 2b which show the infection of the Vero cells under the same conditions as the positive with the exception of plant treatment and combination with the virus. Infecting with the combination, like in the neutralization assay which showed antiviral activity, and comparing to the positive controls allows for confirmation or elimination of the possibility the plant extract is interfering with the attachment of the virus and causing the antiviral activity in that manner. Images 2a and 2b show the pinpoint areas of fluorescence at similar quantities in the field of view indicating the presence of attachment in the treatment groups as well as the positive controls.

Chapter 5

Discussion

The African plant, *Alchornea cordifolia* (Pennah), has extensive ethnomedicinal uses including antibacterial, antifungal, and antiplasmodial. There have been limited studies into the antiviral capabilities of this plant extract, but the ones done have shown promise of antiviral activity in Pennah extract. In this experiment the water and methanol extracts of crude Pennah were screened for antiviral activity against herpes simplex virus type 2 (HSV2) in Vero cell lines. Both extracts were screened for antiviral properties by the use of plaque reduction assays. The plaque reduction assay used for screening was a post-adsorption assay. Post-adsorption assays allow for the binding of the HSV2 virions to the Vero cell before application of extract. This demonstrates whether or not the extract is viable as an antiviral following initial attachment of the virus. The idea behind this approach is that once the virus is allowed to infect cells prior to the application of any potential antiviral compound(s), any reduction in the number of plaques must be due to the interference of the applied compound(s) with any of the steps of virus replication inside infected cells (uncoating, replication, assembly, maturation, and release). The methanol extract exhibited higher plaque reduction results and more effective antiviral capabilities than the water extract, though the water extract also showed antiviral activity against HSV2. The Pennah MeOH extract was then further studied to gain a preliminary understanding of the effect it has as an antiviral on the viral replication cycle, and further investigation into the fraction that is most effective against HSV2 in an attempt to uncover the active principle.

Continued screening using post-adsorption assays was the method of distinguishing which fraction of the Pennah MeOH extract contained the most antiviral compounds effective

against HSV2. A non-polar C-18 column was used to separate Pennah MeOH extract into several subfractions by eluting with H₂O followed by various water-methanol mixtures of increasing methanol strength (10% -100% in 10% increments). Each fraction was collected and screened to determine which was the most effective as an antiviral; the 30% MeOH (30% Pennah MeOH) fraction displayed the most antiviral activity against HSV in our basic screening methods. The 20% MeOH fraction also showed appreciable amount of reduction, but not as effective as the 30% Pennah MeOH fraction. This could be of further interest to investigate the properties and components of both fractions in future research. Now that the fraction of the extract was narrowed down further from crude methanol extract, experiments to gain a better idea of how the extract was interfering with the viral replication cycle were undertaken, but not completed.

A basic understanding of the viral replication cycle of HSV2 should be established before moving forward to recognize the possible ways Pennah is interfering and exhibiting antiviral activity. In order to produce infectious viral particles, the biochemical steps of HSV2 replication need to proceed without disruption. There are multiple steps at which the extract could be interrupting the replication of HSV2. The viral particles attach to the cell, this step requiring no exchange of energy and is not dependent on temperature. After attachment and binding of the viral particle to the host cell, in this case Vero cells, the virion must penetrate the cellular membrane and release the genome containing capsid into the cytoplasm. The viral particle is capable of attaching to the Vero cell without further penetration to occur resulting in a possible point of interference by the extract. Following penetration, the capsid is transported to the nucleus where uncoating occurs and the genetic information is inserted into the nucleus to allow for replication. Along the way multiple tegument proteins play a role in the process. Once the genome and proteins are replicated and translated the assembly occurs in the nucleus. After

release from the nucleus maturation occurs in the cytoplasm where the viral particle gets enveloped and exits the cell by exocytosis (14, 21, 43, 45).

Assessing the capabilities of Pennah as an antiviral and the ways in which it remained effective was done by varying types of plaque assays. The dependent factor in most of the assays was the factor of time. The point at which the virus and extract come in contact, and whether or not the application of extract occurred post infection, or pre-infection, were considered when experimenting with the extract and HSV2 on Vero cells. Treating with the extract at different time intervals began illustrating the capabilities of the plant as an antiviral. When discussing the antiviral activity and plaque reduction of this fraction the concentration of 125 µg/mL of 30% Pennah MeOH will be the referenced results due to its higher degree of reproducibility throughout assays. From here forward all percentage of plaque reduction in reference to an experiment will be with treatment of the concentration 125 µg/mL of 30% Pennah MeOH, unless specified otherwise.

The pre-infection assay showed some antiviral activity at 28.8%, which is less than half of what shown when compared to a post-infection treatment assay (64.5%). Though there is some antiviral activity present in the resulting pre-treatment of cells overnight, it can be attributed to a few factors. The wash of the cells after incubation with the plant overnight may not have been fully effective at washing all of the present plant extract off the cells, possibly leaving some extract behind to interact with the virus. This would be reflected in the lessened plaque reduction in comparison to post attachment exposure and continued treatment for the duration of the assay. In addition, further experimentation showed comparable plaque reduction in neutralization assays and post-adsorption assays suggesting the extract is associating with the virus, not the cells themselves, as an antiviral.

The neutralization assay showed 61.7% of free virus was neutralized by 30% Pennah MeOH when combined and incubated pre-infection. This data suggests that the direct application of the extract on virus particles inactivates/damages the free virions in some way interrupting future events in the viral replication cycle, especially the attachment step and to a lesser extent, the penetration. Attachment was considered the step interrupted at first, but through immunofluorescence assays this was disproved. Attachment is not temperature dependent, but penetration is. Through application of high concentration virus to cells under ice-cold conditions and maintained at this temperature to allow virus attachment which can be visualized with indirect immunofluorescence as attached virions could be seen as fluorescing specks, dots or clumps on infected cell surface. Micrographs of this assay depicted small pinpoint areas of fluorescence indicating viral attachment did occur. Since attachment was indicated through an indirect immunofluorescence of a neutralization assay this means that another step of the viral replication cycle is disrupted by the extract, ruling out attachment as the affected step.

Post-adsorption assay shows the effectiveness of the extract as an antiviral against HSV2 in Vero cells after the virus has time to attach and in the presence of the extract for the duration of the incubation and assay. At concentrations higher than 500 $\mu\text{g/mL}$ the extract shows slight toxicity to the cells, the most being at 1000 $\mu\text{g/mL}$. Though the methanol extract may show some slight toxicity at these concentrations it does prove to be extremely effective as an antiviral agent. Even at 250 $\mu\text{g/mL}$ the plaque reduction is 97.8%, which is almost no plaque forming units. At concentrations as low as 7.8125 $\mu\text{g/mL}$ the extract still exhibits antiviral abilities against HSV2 at a plaque reduction of 11.2%. The decrease in plaque reduction as a function of decrease in concentration shows that the components of the plant and how much is available reflects in the effectiveness of the treatment. This demonstrates that the extract is causing the

plaque reduction. This is also shown in the results of the immunofluorescence assay comparing micrographs of cells treated with 250 $\mu\text{g/mL}$ and cells treated with 125 $\mu\text{g/mL}$ at 28 hours post infection. The higher plaque reduction the post-adsorption assays showed was reflected in these images indicating less cells per field of view fluorescing as a way of showing less spread and infection in the cells treated with 250 $\mu\text{g/mL}$.

Further exploration into the part of the viral replication cycle affected was done by performing time sensitive post-adsorption assays. At 1 hour post-infection the viral particles have attached. At 2 hours post-infection the virion has penetrated the cells and are located within the cells. At 8 hours post-infection viral particles are assembling the proteins and genetic material that are being replicated and translated, and not likely that any have exited the cells. At 10-12 hours the assembled virions start releasing, and by 24 hours the total yield is typically released. The new viral particles then infect surrounding cells and the cycle continues until total cell death, or for experimental purposes until the cells are fixed (48 hours when countable plaques have developed). Adding the 30% Pennah MeOH to the cells following 2 hours post-infection and 8 hours post-infection proved almost as high plaque reduction as addition to the cells 1 hour post-infection (average 55.75%). Removing the treatment at 2 and 8 hours post-infection shows substantial decrease in plaque reduction at a low average 9.85% reduction. That means when the extract was applied at either 2 or 8 hours there was over a 50% reduction in plaques compared to cells not treated with the extract at a concentration of 125 $\mu\text{g/mL}$. When the extract was applied 1 hour post-infection, then removed 2 hours or 8 hours later less than a 10% reduction in plaques was observed compared to positive controls with no extract (125 $\mu\text{g/mL}$) applied. These results were supported with micrographs taken of immunofluorescence assays depicting these experiments. Micrographs showed less infected (fluorescing) cells with the 2 and

8 hour addition groups than the positive controls. The 2 and 8 hour extract added micrographs were comparable to micrographs taken of cells with treatment added 1 hour post-infection and left on until cells were fixed. This reflects the very similar plaque reduction percentages seen with countable plaque assays (55.75% compared to 64.5%). The micrographs of removal of treatment at 2 and 8 hours closely resembled and showed no apparent differences to the positive control images, which was supported the limited plaque reduction seen in the assays performed (9.85% compared to 64.5%). This set of data is important in drawing conclusions to what part of the replication cycle is thought to be interfered with by the extract.

Through indirect immunofluorescence and neutralization assays it has been determined that attachment of the virus is not interrupted by the presence of the extract. Based on the time sensitive assays, and considering neutralization of free particles showing comparable plaque reduction, the viral particles need to be exposed to the plant extract to be effective before attachment or penetration. When considering the assays performed it is easy to think the extract is interrupting attachment, but this is proven false with indirect immunofluorescence. Something important to note is that initial infection and production of viral particles does not encompass the entire scope of the HSV2 infection and plaque forming process. Plaques are formed by infection of a Vero cell that results in virulent viral particles and cell death, that then infect the surrounding Vero cells and produce more infectious virions and cause more cell death, and so on until a visible plaque is formed on the flask. That means enough cells in an area have to go through subsequent infections from the initial starting cell infection to create a plaque visible to the eye. Therefore, initial infection may occur and produce subsequent viral particles that are then affected by the plant extract's antiviral properties. Taking this possibility into consideration presents challenges when determining which part of the viral replication cycle is being disrupted.

Through the assays it is clear that the extract interacts with the virus, and when the plant extract was not applied before attachment, and was not available for interaction for the subsequent viral particles, little plaque reduction was observed (removing after 2 and 8 hours after attachment). Though the extract may not prevent attachment, it may interfere with some of the surface proteins necessary for penetration to occur. The viral particle binds due to interactions between the receptors on the cell's surface and glycoproteins on the envelope of the virion. After this binding, more glycoproteins (gD, gH/L) are involved to allow for penetration. Once gC binds, gD must bind to one of its co-receptors (HveA/HVEM, Nectin-1, or 3-O sulfated heparan sulfate) to allow for the fusion of membranes and viral entry (1, 2, 43). The extract could interfere with this process and result in viral attachment, but no subsequent penetration, therefore halting the infection by that virion. The viral particle could also associate with the extract before attachment and still penetrate leading to disruption of other early replication events such as uncoating, replication, or assembly. Limited time and resources did not allow for further analysis in determining the specific viral replication cycle event being interfered with resulting in the antiviral activity.

Two techniques were used to analyze the composition of the extract. Chemical detection methods were used to determine what class of compounds may be present in the extract. The methods used determine the presence of large quantities of the chemical classes and do not necessarily detect the presence of every single type of compound within the class. More directed and advanced methods would be necessary to grasp every compound present in each class. The 30% Pennah MeOH extract tested positive for alkaloids, phenols, and terpenoids with the methods used. Testing for flavonoids gave a negative result in this experimentation. Alkaloids, terpenoids, and phenols have all been found to contain compounds that have acted as antivirals

making their presence in the extract of interest possibly contributing to the antiviral activity or containing the compound that may be causing the antiviral activity (4, 11, 42).

Preliminary thin-layer chromatography was attempted to distinguish how many major components of the plant extract were present. TLC plates were run with crude methanol extract and 30% Pennah MeOH alone and on the same plate to better compare. In the comparison there is one bright band visible under the long wavelength of UV light that shows up in both extracts. Another plate was run with four lanes of varying concentrations of 30% Pennah MeOH and was run in the solvent twice to try to further resolve the bands and possibly uncover bands hidden within areas already noted. These bands indicate groupings of compounds with similar mobility on the silica plate under the certain conditions, such as the solvent used to run them in the column. This was done to analyze how many major components may be present. This resulted in four distinct bands. The bands may indicate like components, but they do not necessarily indicate the presence of a single compound in each band. Each band indicates components of the extract that had similar mobility on the silica plate. Additional advanced analysis methods would need to be applied to the extract to gain a further understanding of the composition of 30% Pennah MeOH.

Conclusions

The research conducted in this study has established beyond doubt that *Alchornea cordifolia* possesses antiviral properties against HSV2 in Vero cells. The fraction of crude methanol extract that exhibited the highest plaque reduction was the 30% MeOH fraction. Based on varying time sensitive plaque assays, coupled with indirect immunofluorescence, the research suggests that the antiviral activity observed occurs during the events following attachment, possibly at the penetration step. These results do not allow for definitive answers surrounding the specific step the antiviral activity occurs. Due to time and resource limitations more research is necessary to determine the specific event that is disrupted.

Preliminary thin-layer chromatography suggests the presence of four main groupings of similar components based on mobility. Chemical class detection methods determined the presence of alkaloids, phenols, and terpenes, each class containing potential antivirals.

Additional work is necessary to determine whether a single compound, or multiple compounds, are causing the antiviral activity observed. Further research concentrating on the identification, and consequent isolation, of active compounds found in *Alchornea cordifolia* is essential for the analysis of the mechanism of action this extract is exhibiting in Vero cells on HSV2.

Identification of this compound and mode of action could result in useful medicinal development of a new antiviral agent for the treatment of the HSV2, as well as possible relief concerning other ailments.

References

1. Agelidis, A. M., & Shukla, D. (2015). Cell entry mechanisms of HSV: What we have learned in recent years. *Future Virology*, 10(10), 1145-1154. doi:10.2217/fvl.15.85
2. Akhtar, J., & Shukla, D. (2009). Viral entry mechanisms: Cellular and viral mediators of herpes simplex virus entry. *FEBS Journal*, 276(24), 7228-7236. doi:10.1111/j.1742-4658.2009.07402.x
3. Álvarez, D. M., Castillo, E., Duarte, L. F., Arriagada, J., Corrales, N., Farías, M. A., . . . González, P. A. (2020). Current Antivirals and Novel Botanical Molecules Interfering With Herpes Simplex Virus Infection. *Frontiers in Microbiology*, 11. doi:10.3389/fmicb.2020.00139
4. Astani, A., Reichling, J., & Schnitzler, P. (2009). Comparative study on the antiviral activity of selected monoterpenes derived from essential oils. *Phytotherapy Research*. doi:10.1002/ptr.2955
5. Ayisi, N.K., Nyadedzor, C., 2003. Comparative in vitro effects of AZT and extracts of *Ocimum gratissimum*, *Ficus polita*, *Clausena anisata*, *Alchornea cordifolia*, and *Elaeophorbia drupifera* against HIV-1 and HIV-2 infections. *Antivir. Res.* 58 (1), 25–33.
6. Bickford, J., Barton, S.E., Mandalia, S. Chronic genital herpes and disclosure.... The influence of stigma. (2007). *International Journal of STD & AIDS*, 18(9), 589–592. doi:10.1258/095646207781568484
7. Boehmer, P., & Nimonkar, A. (2003). Herpes Virus Replication. *IUBMB Life (International Union of Biochemistry and Molecular Biology: Life)*, 55(1), 13-22. doi:10.1080/1521654031000070645

8. Boniface, P. K., Ferreira, S. B., & Kaiser, C. R. (2016). Recent trends in phytochemistry, ethnobotany and pharmacological significance of *Alchornea cordifolia* (Schumach. & Thonn.) Muell. Arg. *Journal of Ethnopharmacology*, 191, 216-244.
doi:10.1016/j.jep.2016.06.021
9. Boissière, S. L., Izeta, A., Malcomber, S., & O'hare, P. (2004). Compartmentalization of VP16 in Cells Infected with Recombinant Herpes Simplex Virus Expressing VP16-Green Fluorescent Protein Fusion Proteins. *Journal of Virology*, 78(15), 8002-8014.
doi:10.1128/jvi.78.15.8002-8014.2004
10. Cernik, C. (2008). The Treatment of Herpes Simplex Infections An Evidence-Based Review [Abstract]. *Archives of Internal Medicine*, 168(11), 1137.
doi:10.1001/archinte.168.11.1137
11. Chávez, J. H., Leal, P. C., Yunes, R. A., Nunes, R. J., Barardi, C. R., Pinto, A. R., . . . Zanetti, C. R. (2006). Evaluation of antiviral activity of phenolic compounds and derivatives against rabies virus. *Veterinary Microbiology*, 116(1-3), 53-59.
doi:10.1016/j.vetmic.2006.03.019
12. Chinchar, V. (1999). Replication Of Viruses. *Encyclopedia of Virology*, 1471-1478.
doi:10.1006/rwvi.1999.0245
13. Connolly, S. A., Landsburg, D. J., Carfi, A., Wiley, D. C., Eisenberg, R. J., & Cohen, G. H. (2002). Structure-Based Analysis of the Herpes Simplex Virus Glycoprotein D Binding Site Present on Herpesvirus Entry Mediator HveA (HVEM). *Journal of Virology*, 76(21), 10894-10904. doi:10.1128/jvi.76.21.10894-10904.2002

14. Copeland, A. M., Newcomb, W. W., & Brown, J. C. (2008). Herpes Simplex Virus Replication: Roles of Viral Proteins and Nucleoporins in Capsid-Nucleus Attachment. *Journal of Virology*, 83(4), 1660-1668. doi:10.1128/jvi.01139-08
15. Damian, C. O., Festus, B. C., Sylvester, C. N., & Charles, C. E. (2018). Anti-respiratory syncytial virus activities of leaf extracts of *Alchornea cordifolia* and *Alchornea floribunda*. *African Journal of Pharmacy and Pharmacology*, 12(8), 97-105. doi:10.5897/ajpp2018.4890
16. Davison, A. J. (2010). Herpesvirus systematics. *Veterinary Microbiology*, 143(1), 52-69. doi:10.1016/j.vetmic.2010.02.014
17. Giday, M., Asfaw, Z., Elmqvist, T., & Woldu, Z. (2003). An ethnobotanical study of medicinal plants used by the Zay people in Ethiopia. *Journal of Ethnopharmacology*, 85(1), 43-52. doi:10.1016/s0378-8741(02)00359-8
18. Grinde, B. (2013). Herpesviruses: Latency and reactivation – viral strategies and host response. *Journal of Oral Microbiology*, 5(1), 22766. doi:10.3402/jom.v5i0.22766
19. Gupta, R., & Wald, A. (2006). Genital herpes: Antiviral therapy for symptom relief and prevention of transmission [Abstract]. *Expert Opinion on Pharmacotherapy*, 7(6), 665-675. doi:10.1517/14656566.7.6.665
20. Huang, K. (2006). Inhibition of hepatitis B virus production by *Boehmeria nivea* root extract in HepG2 2.2.15 cells. *World Journal of Gastroenterology*, 12(35), 5721. doi:10.3748/wjg.v12.i35.5721
21. Ibáñez, F. J., Farías, M. A., Gonzalez-Troncoso, M. P., Corrales, N., Duarte, L. F., Retamal-Díaz, A., & González, P. A. (2018). Experimental Dissection of the Lytic

- Replication Cycles of Herpes Simplex Viruses in vitro. *Frontiers in Microbiology*, 9. doi:10.3389/fmicb.2018.02406
22. Johnson, D. C., & Baines, J. D. (2011). Herpesviruses remodel host membranes for virus egress [Abstract]. *Nature Reviews Microbiology*, 9(5), 382-394. doi:10.1038/nrmicro2559
 23. Lint, A. V., & Knipe, D. (2014). Herpesviruses [Abstract]. *Reference Module in Biomedical Sciences*. doi:10.1016/b978-0-12-801238-3.02509-5
 24. Marein A. W. P. de Jong, Lot de Witte, Maureen E. Taylor, Teunis B. H. Geijtenbeek. (2010). Herpes Simplex Virus Type 2 enhances HIV-1 susceptibility by affecting Langerhans cell function. *The Journal of Immunology*, 185(3), 1633-1641. doi:10.4049/jimmunol.0904137
 25. McQuillan G., Kruszon-Moran D., Flagg EW, Paulose-Ram R. (2018, February). *Prevalence of herpes simplex virus type 1 and type 2 in persons aged 14–49*. United States, 2015–2016. NCHS Data Brief, no 304. Hyattsville, MD: National Center for Health Statistics.
 26. Mettenleiter, T. C. (2002). Herpesvirus Assembly and Egress. *Journal of Virology*, 76(4), 1537-1547. doi:10.1128/jvi.76.4.1537-1547.2002
 27. Mettenleiter, T. C., Müller, F., Granzow, H., & Klupp, B. G. (2012). The way out: What we know and do not know about herpesvirus nuclear egress. *Cellular Microbiology*, 15(2), 170-178. doi:10.1111/cmi.12044
 28. Mindel, A., Marks, C. Psychological Symptoms Associated with Genital Herpes Virus Infections. *CNS Drugs* 19, 303–312 (2005). doi:10.2165/00023210-200519040-00003
 29. Mossman, K. L., Sherburne, R., Lavery, C., Duncan, J., & Smiley, J. R. (2000). Evidence that Herpes Simplex Virus VP16 Is Required for Viral Egress Downstream of the Initial

- Envelopment Event. *Journal of Virology*, 74(14), 6287-6299. doi:10.1128/jvi.74.14.6287-6299.2000
30. Mukhtar, M., Arshad, M., Ahmad, M., Pomerantz, R. J., Wigdahl, B., & Parveen, Z. (2008). Antiviral potentials of medicinal plants. *Virus Research*, 131(2), 111-120. doi:10.1016/j.virusres.2007.09.008
31. Muylaert, I., Tang, K., & Elias, P. (2011). Replication and Recombination of Herpes Simplex Virus DNA. *Journal of Biological Chemistry*, 286(18), 15619-15624. doi:10.1074/jbc.r111.233981
32. Nikolic, D. S., & Piguet, V. (2010). Vaccines and Microbicides Preventing HIV-1, HSV-2, and HPV Mucosal Transmission. *Journal of Investigative Dermatology*, 130(2), 352-361. doi:10.1038/jid.2009.227
33. Nolkemper, S., Reichling, J., Sensch, K. H., & Schnitzler, P. (2010). Mechanism of herpes simplex virus type 2 suppression by propolis extracts. *Phytomedicine*, 17(2), 132-138. doi:10.1016/j.phymed.2009.07.006
34. Payne, S. (2017). Family Herpesviridae [Abstract]. *Viruses*, 269-278. doi:10.1016/b978-0-12-803109-4.00034-9
35. Richman, D. D., Whitley, R. J., & Hayden, F. G. (2017). Herpes Simplex Viruses. In *Clinical virology* (4th ed., pp. 415-445). Washington, DC: ASM Press. doi:10.1128/9781555819439.ch20
36. Roizman, B., & Zhou, G. (2015). The 3 facets of regulation of herpes simplex virus gene expression: A critical inquiry. *Virology*, 479-480, 562-567. doi:10.1016/j.virol.2015.02.036

37. Salvaggio, M. R., & Gnann, J. W., Jr. (2017). 153 Drugs for Herpesvirus Infections. In J. Cohen, W. G. Powderly, & S. M. Opal (Eds.), *Infectious diseases*(8th ed., Vol. 2). Amsterdam: Elsevier. doi:10.1016/B978-0-7020-6285-8.00153-2
38. Schiffer, J. T., & Corey, L. (2015). 138 Herpes Simplex Virus. In *Mandell, Douglas, and Bennett's principles and practice of infectious diseases*(8th ed., Vol. 2, p. 1713). Philadelphia, PA: Elsevier. doi:10.1016/B978-1-4557-4801-3.00138-7
39. Schiffer, J. T., Wald, A., Selke, S., Corey, L., & Magaret, A. (2011). The Kinetics of Mucosal Herpes Simplex Virus–2 Infection in Humans: Evidence for Rapid Viral-Host Interactions. *The Journal of Infectious Diseases*,204(4), 554-561.
doi:10.1093/infdis/jir314
40. Steiner, I., & Benninger, F. (2018). Manifestations of Herpes Virus Infections in the Nervous System. *Neurologic Clinics*,36(4), 725-738. doi:10.1016/j.ncl.2018.06.005
41. Strasfeld, L., & Chou, S. (2010). Antiviral Drug Resistance: Mechanisms and Clinical Implications. *Infectious Disease Clinics of North America*,24(2), 413-437.
doi:10.1016/j.idc.2010.01.001
42. Thawabteh, A., Juma, S., Bader, M., Karaman, D., Scrano, L., Bufo, S. A., & Karaman, R. (2019). The Biological Activity of Natural Alkaloids against Herbivores, Cancerous Cells and Pathogens. *Toxins*,11(11), 656. doi:10.3390/toxins11110656
43. Tognarelli, E. I., Palomino, T. F., Corrales, N., Bueno, S. M., Kalergis, A. M., & González, P. A. (2019). Herpes Simplex Virus Evasion of Early Host Antiviral Responses. *Frontiers in Cellular and Infection Microbiology*,9.
doi:10.3389/fcimb.2019.00127

44. Weller, Sandra K., & Donald M. Coen. "Herpes simplex viruses: mechanisms of DNA replication." *Cold Spring Harbor perspectives in biology* 4.9 (2012): doi:a013011
45. Whitley, R. J. (1996). Chapter 68 Herpesviruses (S. Baron, Ed.). In *Medical Microbiology* (4th ed.). Galveston, TX: University of Texas Medical Branch at Galveston. Retrieved from <https://www.ncbi.nlm.nih.gov/books/NBK8157/>
46. Wiedbrauk, D. L. (2010). Herpes Simplex Virus. *Molecular Diagnostics*, 453-460. doi:10.1016/b978-0-12-369428-7.00037-9
47. World Health Organization. (2020, May 1). *Herpes Simplex Virus*. Retrieved from: <https://www.who.int/news-room/fact-sheets/detail/herpes-simplex-virus>